BIOCHEMICAL CHARACTERIZATION OF TWO WHEAT PHOSPHOETHANOLAMINE N-METHYLTRANSFERASE ISOFORMS WITH DIFFERENT SENSITIVITIES TO INHIBITION BY PHOSPHATIDIC ACID

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RUNNING TITLE

analysis of two wheat phosphoethanolamine methyltransferases

In plants the triple methylation of phosphoethanolamine to phosphocholine catalysed by phosphoethanolamine N-methyltransferase (PEAMT) is considered a rate limiting step in the de novo synthesis of phosphatidylcholine. Besides being a major membrane phospholipid, phosphatidylcholine can be hydrolysed into choline and phosphatidic acid. Phosphatidic acid is widely recognized as a second messenger in stress signalling and choline can be oxidized within the chloroplast to yield the putative osmoprotectant glycine betaine. Here we describe the cloning and biochemical characterization of a second wheat PEAMT isoform that has a four times higher specific activity than the previously described WPEAMT/TaPEAMT1 enzyme and is less sensitive to product inhibition by S-adenosyl homocysteine, but more sensitive to inhibition by phosphocholine. Both enzymes follow a sequential random Bi Bi mechanism and show mixed-type product inhibition patterns with partial inhibition for TaPEAMT1 and a strong non-competitive component for TaPEAMT2. An induction of TaPEAMT protein expression and activity is observed after cold exposure, ahead of an increase in gene expression. Our results demonstrate direct repression of in vitro enzymatic activities by phosphatidic acid for both enzymes, with TaPEAMT1 being more sensitive than TaPEAMT2 in the physiological concentration range. Other lipid ligands identified in protein-lipid overlays are phosphoinositide mono- as well as some di-phosphates and cardiolipin. These results provide new insights into the complex regulatory circuits of phospholipid biosynthesis in plants and underline the importance of head group biosynthesis in adaptive stress responses.

Phosphatidylcholine (PC1), one of the major plasma membrane phospholipids in most eukaryotes, has been shown to undergo changes in turnover in response to various environmental stresses in plants (for review see (1,2)) and has a major impact on cell proliferation and apoptosis in mammals (for review see (3)). In single and multicellular organisms, biosynthetic routes leading to the production of PC are diverse and seem to depend on the availability of precursors for the synthesis of the hydrophilic head group, phosphocholine (P-Cho, see suppl. figure 1). Plants use the free phosphobase methylation pathway and can catalyse the conversion of serine to ethanolamine via a unique serine decarboxylase (4). Ethanolamine undergoes phosphorylation followed by the triple methylation of the free phosphobase to P-Cho, catalysed by PEAMT (5,6). P-Cho can then be used as the head group to form PC by the two enzymes P-Cho cytidylyltransferase and CDP-choline:diacylglycerol phosphotransferase, that are part of the Kennedy
pathway in eukaryotes (7). Genetic evidence was recently presented in Plasmodium that the knockout mutant of the PfPMT gene is unable to synthesise PC in the absence of an external choline supply (8). Plasmodium therefore lacks the enzyme phosphatidylethanolamine methyltransferase found in fungi and mammals. As higher plants lack that enzyme too and are choline autotroph, the PEAMT catalysed reactions seem likely to be the sole entry point for P-Cho and choline into plant metabolism (9-11). Any choline kinase activity can therefore be seen as a recycling or feedback regulatory function rather than being involved in de novo biosynthesis of P-Cho and PC (12,13). Plants have evolved two different ways of synthesizing choline: Chenopodiaceae such as spinach and sugarbeet are capable of producing it directly from ethanolamine via ethanolamine kinase, PEAMT and P-Cho phosphatase catalysis, and use it largely for the biosynthesis of glycine betaine (GlyBet, (5,14). Gramineae like wheat and barley, however, seem to produce choline, and therefore subsequently GlyBet, through PC hydrolysis (10,15). One explanation for this could be the low activity of P-Cho phosphatase in these species, for which the identity of the encoding gene is still somewhat elusive (12).

GlyBet is thought to act as a compatible solute or osmoprotectant under various abiotic stresses ranging from drought and salinity to cold (16-18). The ability of different genotypes to withstand these conditions has been shown to correlate with their ability to synthesize GlyBet (19-21). This compound acts in the chloroplast to protect photosystem II and other protein complexes, as well as to maintain membrane integrity and scavenge reactive oxygen species, but also to reduce the extent of K⁺ loss in response to salinity and oxidative stress (see (22) for review). Interestingly, some of these functions overlap with those proposed for alterations in cell membrane phospholipid composition that have been observed under cold or drought stress (23-26). It is therefore not entirely clear whether GlyBet accumulation is a protective mechanism in itself, a symptom of stress susceptibility (27) or merely a consequence of increased phospholipid turnover (10,25).

Due to its central position in the phospholipid and GlyBet synthetic pathways the role of PEAMT in plants’ response to cold or salinity has been intensively studied (14,28,29). While PEAMT has been proposed to be one important bottle-neck for the de novo synthesis of choline, GlyBet and PC (30), other enzymes such as S-adenosylmethionine (SAM) synthetase, S-adenosylhomocysteine (SAH) hydrolase, choline kinase and CTP:P-Cho cytidylyltransferase have also been shown to have increased activities under these abiotic stress conditions (13,29,31,32).

Individual PEAMT enzymes from spinach, wheat and Arabidopsis have been characterized at the molecular level (28,33,34). They are all putative cytosolic enzymes composed of two methyltransferase domains. In the spinach SoPEAMT and wheat WPEAMT/TaPEAMT1 enzymes the N-terminal domain catalyses the first methylation step, while the C-terminal half is only capable of catalysing the second and third methylations. Although more than one PEAMT isoform has been identified in maize, rice and Arabidopsis (13,35), no detailed genomic or biochemical comparison of those isoforms in any species has been undertaken so far.

Here we report the molecular and biochemical characterisation of two phosphoethanolamine N-methyltransferase isoforms in wheat. They show differential transcriptional regulation in response to cold acclimation and pronounced differences in their enzymatic properties that enable them to operate under different metabolic conditions. The consequences of these differences in enzymatic function for the plant’s adaptability to different environments as well as for genetic engineering will be discussed.

**Experimental Procedures**

**Materials** – All reagents, unless stated otherwise, were purchased from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, AUS).

**Plant Material – Triticum aestivum** cv. Egret plants were grown in sterile vermiculite grade 3 under 16-h photoperiod at 270 μmol m⁻² sec⁻¹ photon flux density, 24°C (21°C at night) and 80 % relative humidity. Plants were watered daily with 1/3 Hoagland’s solution (36) by replacing the amount of water lost through transpiration and direct evaporation. For the cold treatment 7-day old seedlings were transferred to a cabinet set up to match the same conditions as above, but running at 7°C (day) and 4°C (night). 1, 2 and 6 days later roots and shoots were harvested from both controls.
and cold treated plants, immediately frozen in liquid N2 and then stored at -80ºC.

Expression and Purification of His-tagged Wheat PEAMT Proteins – Following PCR amplification on a wheat cv. Egret cDNA library, the wheat TaPEAMT1 open reading frame was cloned into the E.coli expression vector pET28a (Novagen / Merck Pty., Kilsyth, VIC, AUS) using the following primers: 5’- AACATATGGACACCACCTACCGTCGTC-3’ containing a 5’ NdeI restriction site and 5’-AAGGATCTCCTACCTGTTCGCATGAAGCAG-3’ containing a 5’ BamHI restriction site. The resulting overexpression plasmids were sequenced and transformed into E.coli Rosetta (DE3) pLysS (Novagen). Recombinant His-tagged PEAMT proteins were purified to near-homogeneity by NiNTA affinity chromatography (see suppl. figure 2A). Protein purity was estimated by SDS-PAGE followed by Coomassie staining and protein concentration was determined using Bradford’s Reagent (BioRad Laboratories, Regents Park, NSW, AUS). Aliquots of the purified recombinant PEAMT proteins were stored at -80ºC.

Antibody Production – Recombinant TaPEAMT1 protein was eluted from the HiTrap column as before and exchanged into 1xPBS using a PD-10 column. This antigen solution (1 mg/ml) was injected into rabbits using standard protocols for antibody production. The α-TaPEAMT1 polyclonal antibody detects recombinant TaPEAMT1 and TaPEAMT2 proteins with comparable affinity (data not shown).

Protein Extraction and Immunoblotting – Protein extraction and immunoblotting were carried out as described previously (37). α-TaPEAMT1, α-RbcL and α-TCTP (37) primary antibodies were used as 1:1000, 1:10000 and 1:5000 dilutions, respectively. Anti-rabbit IgG AP conjugate (Promega Corporation, Annandale, NSW, AUS) was used in a 1:10000 dilution. Chemiluminescent detection was performed using the Western-Star Immunodetection System according to the manufacturer’s instructions (Applied Biosystems, Scoresby, VIC, AUS) and a CCD camera system (VersaDoc 3000, Bio-Rad).

Quantitative Real-Time RT-PCR – mRNA isolation and reverse transcription using magnetic oligo(dT)25 coated beads (Dynabeads®, Invitrogen Australia Pty Limited, Mount Waverly, VIC, AUS) were carried out as described previously (38). qPCR and C value determination were carried out using an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) and accompanying software according to the manufacturer’s instruction. Reactions contained: 2.5 µl bead-bound cDNA (0.5 ng mRNA equivalent), 2.5 µl primer mix (1.2 µM each) and 5 µl 2x Power SYBR® Green PCR Master Mix (Applied Biosystems). After each run a melting curve analysis was performed to verify target specific product amplification. Primers used for specific amplification of target transcripts were: TaPEAMT1 (Acc.No. AY065971) 5’-GACGCACCGAGCTCCGCGCA-3’ and 5’-GCCGTCAGCGGGTCTCATAGGATAG-3’, TaPEAMT2 (Acc.No. FJ803924) 5’- AAAAACTTGGGTAAGGTCTCTTAATCG-3’ and 5’-CTTAATGTATGCGGAAAACCTCTCCATCGAG-3’. The following primers were used to detect reference gene expression: TaAPT1 (Acc.No. U22442) 5’-GGCCCTTCAGGCTTGACCAGAT-3’ and 5’-TAAAATTACTGGGCTAGAAAGACTCT-3’, TaEF1α (Acc.No. M90077) 5’- CGCATATACACGGCGCTAAACG-3’ and 5’-CGCATATACACGGCGCTAAACG-3’.

Preparation of Lipid Vesicles – Liposomes were prepared according to Potocky et al. (39). 3-snap phosphatidic acid from egg yolk or L-alpha-phosphatidylcholine from soybean (Sigma Aldrich) were dissolved in chloroform / methanol (2:1, v/v). After evaporation under a dry nitrogen stream the lipid film was hydrated in MilliQ water for one hour followed by 30 min sonication in a water bath at a temperature above the gel-liquid crystal transition temperature of the individual lipid. Aliquots of 100 mM stock solutions of these small, unilamellar vesicles were stored in glass vials at -20ºC.
Phosphoethanolamine N-methyltransferase Assay
– Unless stated otherwise, all purification steps were carried out at 4°C. 100 mg plant tissue ground under liquid N2 was homogenized in 1.5 volumes of 50 mM HEPES pH 7.8, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 5 mM DTT and 0.5 mM PMSF buffer. After centrifugation 120 µl of supernatant was passed through a desalting spin column (Pierce / Thermo Fisher Scientific Inc., Rockford, IL, USA) pre-equilibrated in 4 volumes of 50 mM HEPES pH 7.8, 1 mM EDTA, 10% (v/v) glycerol, 5 mM DTT and 0.5 mM PMSF. Protein amounts were determined using Bradford’s reagent (BioRad). 80 to 160 µg of leaf protein extract and 60 µg of root protein extract were incubated in 50 mM HEPES pH 8.6, 1 mM EDTA, 800 µM phosphoethanolamine (P-EA) and 2000 µM SAM (60:1 ratio of cold SAM over 14C-SAM / S-adenosyl-L-[methyl-14C]- methionine with an average specific activity of 2 GBq/mmol, GE Healthcare Biosciences Pty. Ltd., Rydalmere, NSW, AUS). For control assays P-EA was omitted. Samples were incubated at 30ºC for 30 min and reactions were stopped by shock-freezing in liquid N2. To separate unincorporated 14C labelled SAM from methylated products, reaction mixes were passed through 2-ml AG 50W-X8 (H+) cation exchanger columns (BioRad) as described by Charron et al. (28) except that samples were eluted twice in 5 ml of 1 N HCl. 1 ml of combined eluates was added to 3 ml of Ultima Gold XR scintillation liquid (Packard BioScience / PerkinElmer Pty. Ltd., Rowville, VIC, AUS) and counted for 20 min in a Tri-Carb 2800TR liquid scintillation analyser (Perkin Elmer) using a 14C quench correction for the amount of HCl in each sample. Elution efficiency of methylated phosphobases was estimated by comparing samples with and without addition of cold SAM. Enzyme Kinetics – In all experiments the reactions were only allowed to proceed to a negligible extent (6 – 7% for TaPEAMT1 and 9 – 14 % for TaPEAMT2 at saturating substrate concentrations) and product formation was linear in response to protein concentration and time. Unless stated otherwise, 1 µg of recombinant TaPEAMT protein was used. The assay conditions and ion exchange chromatography procedures were as mentioned above. To ensure that the column’s cation exchanger binding capacity was not exceeded, assay reactions using high substrate amounts were diluted prior to column loading. For initial velocity studies both substrates were varied in a 5x5 matrix according to Eisenthal and Danson ((40), see table 1). In control reactions either P-EA or SAM were omitted. For product inhibitor studies the corresponding substrate-product pairs, i.e. P-EA and P-Cho or SAM and SAH were varied accordingly, with the concentration of the second substrate kept at saturation (see table 1). For phosphatidic acid (PA) and phosphocholine (PC) inhibition plots phospholipid concentrations were varied while both substrates were used at saturating concentrations. Initial velocity data for the two-substrate kinetics or product inhibitor studies were fitted to the general velocity equations using the Enzyme Kinetics Module for Sigma Plot (SYSTAT, see suppl. figure 4A for equations).

Lipid-Protein Overlay Assay – Binding of recombinant PEAMT protein to phospholipids was analysed according to Dowler et al. (41) using 1 mM stocks of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC) or –phosphate (PA, Echelon Biosciences Inc., Salt Lake City, UT, USA) Spotted membranes as well as commercial membrane lipids (ML) and phosphoinositide (PIP) strips (Echelon Biosciences Inc.) were blocked for 4 h in 0.2% I-block in 1xPBST. Blots were then incubated with 40 to 80 nM of recombinant TaPEAMT protein overnight at 4°C, before lipid-bound protein was detected using a 1:2000 dilution of α-TaPEAMT1 antibody (see above) and 1:10.000 dilution of α–Rabbit IgG AP conjugate (Promega Corporation) followed by chemiluminescence detection according to the manufacturer’s instructions (Applied Biosystems).

RESULTS

Identification of a Second Wheat PEAMT cDNA – Through sequence comparison of the published WPEAMT/TaPEAMT1 cDNA sequence (28), accession no. AY065971, chromosome 1) with available EST data (http://www.gramene.org/, http://www.jcvi.org/ and the UniGene EST collection at http://blast.ncbi.nlm.nih.gov/) we identified a second set of EST sequences distinctly different from the homologous classes of WPEAMT/TaPEAMT1 contigs. This new set of sequences fell into three contig classes likely
representing homeologs from the three wheat genomes. We used the most prominent class of EST sequences to deduce an \textit{in silico} TaPEAMT2 cDNA sequence prediction and to design primers to amplify the cDNA for subsequent analyses. The cloned open reading frame sequence for TaPEAMT2 shows 77\% identity to the WPEAMT/TaPEAMT1 ORF and has been submitted to GenBank, accession number FJ803924. Both cDNAs encode 57 kDa proteins that seem to lack N-terminal signal peptide sequences and therefore most likely localise to the cytoplasm.

\textbf{Phylogenetic Analysis of PEAMT Proteins} – Higher plant PEAMT proteins form two distinct clades for dicotyledonous and monocotyledonous species, clearly separated from the protein sequence for the moss \textit{Physcomitrella patens} and the rather isolated protein sequence from purple sea urchin as well as from the other two clades, one containing three vertebrate proteins from \textit{Xenopus laevis}, pufferfish and zebrafish, the other containing the protein from the apicomplexan parasite \textit{Plasmodium falciparum} and two \textit{C. elegans} proteins, CePMT-1 and -2 (see suppl. figure 3). The proteins in the vertebrate clade are only putative PEAMT orthologs and have not been characterized in any detail yet. The higher plant proteins fall into several distinct groups with the monocot clade forming two branches, one for each of the two isoforms that can be found in nearly all species analysed to date, including the newly identified TaPEAMT2 sequence characterized in this study. This could be interpreted as a sign of some specialisation of enzymatic function within monocotyledons. The dicot clade is structured quite differently, with only one isoform identified for most species to date, with the exception of \textit{Arabidopsis thaliana} (three isoforms, NMT1-3), \textit{Medicago truncatula} and \textit{Vitis vinifera} (two isoforms). \textit{AtNMT1} and \textit{AtNMT2} are located on duplicated segments of chromosomes 3 and 1, respectively, indicating that they have arisen from a very recent gene duplication event (42). A very strong separate branch is formed by known Chenopodiaceae proteins, indicating that some enzyme specialisation occurred for these GlyBet accumulators (5,9,14). There is, however, no general trend for protein sequences of GlyBet accumulators to group together and the dicotyledonous clade is organized instead according to taxonomic groups.

\textbf{Response to Cold Acclimation} – After the identification of a second PEAMT isoform in wheat we probed for functional differences using cold treatments that have been shown to induce PEAMT gene expression in wheat and \textit{Arabidopsis} (13,28). As expected from these published results we were able to detect increases in PEAMT protein and activity levels in leaves within 24 to 48h after transfer to cold, with a 6-fold increase in total PEAMT protein amounts and a 4-fold increase of \textit{in vitro} activity observed after 6 days exposure (figure 1A+B). No PEAMT activity or protein expression has previously been detected in roots (28), but in this study total protein and activity levels in roots were comparable to those in leaves and an increase in total root PEAMT protein (7-fold) and activity (2-fold) was recorded after 6 days of cold acclimation. Analysis by gene specific qRT-PCR showed a differential response of the two TaPEAMT genes with a clear induction of TaPEAMT2 being detectable 6d after transfer, in both leaves and roots, while changes in TaPEAMT1 transcript remained within noise level (figure 1C). It is likely then that the large induction of PEAMT protein expression and \textit{in vitro} activity at that time primarily reflects the induction of the TaPEAMT2 isoform. The absence of TaPEAMT1 gene induction by cold contrasts with an earlier report by Charron et al. based on Northern hybridisation experiments. Differential expression of PEAMT genes has also been reported in \textit{Arabidopsis} under salt stress (13). The absence of significant changes in either PEAMT gene expression at the two-day time point in our experiment when protein expression was already significantly increased in leaves (figure 1B, left panel) is indicative of post-transcriptional or post-translational regulation of PEAMT. In order to assess the physiological significance of these results, we decided to examine the kinetic properties of the two wheat PEAMT isoforms.

\textbf{Biochemical Characterisation} – To date only one isoform from spinach, \textit{Arabidopsis} and wheat have been biochemically characterized (6,28,33,34). These experiments used only partially purified proteins, either isolated from plant material or recombinantly expressed in \textit{E.coli} or yeast. In this study we are comparing the kinetic properties of two different PEAMT isoforms within a plant
species using highly purified recombinant proteins (suppl. figure 2A). After correcting for residual contaminant E.coli proteins in the final desalted protein extracts the protein dependence of PEAMT activity was determined for both isoforms. Specific P-base N-methylation activities were determined from the linear phase of the two curves in suppl. figure 2B. Using physiologically relevant substrate concentrations of 200 μM P-EA and SAM as described earlier (6), we found that TaPEAMT2 is 4-times more active than TaPEAMT1 with specific activities of 2353 ± 122 and 570 ± 62 pkat / mg protein, respectively. The TaPEAMT2 specific activity is quite similar to that reported for the purified native spinach enzyme (6), while the value we obtained for recombinant TaPEAMT1 is about 90-fold higher than reported previously for recombinant WPEAMT from crude E.coli extracts (28).

Initial Velocity Studies – Although apparent K_m values for the two substrates P-EA and SAM have been reported for one recombinant wheat and one recombinant spinach PEAMT enzyme in crude extracts (28,33), no detailed mechanistic study of any plant enzyme has been performed to date. When we used a similar substrate concentration range as in these published analyses in our experiments, apparent K_m values were of the same order as those reported for the recombinant spinach enzyme with 198 ± 16 μM (TaPEAMT1) and 111 ± 12 μM (TaPEAMT2) for P-EA and 396 ± 38 μM (TaPEAMT1) and 212 ± 43 μM (TaPEAMT2) for SAM, and revealed higher affinities of the second wheat isoform for both substrates. It was obvious, however, from these initial measurements that no true substrate saturation had been achieved (data not shown). We therefore undertook a more detailed mechanistic study by varying both substrates simultaneously over a wider concentration range (figure 2 and table 1). While for both enzymes the K_m values for P-EA increased by less than 2-fold, there was a 5-fold decrease in TaPEAMT2 affinity for SAM under saturating compared to non-saturating conditions (data not shown). For TaPEAMT1, however, the affinity for SAM decreased only about 1.5-fold. The favoured kinetic model for both enzymes is that of a sequential random Bi-Bi mechanism (R^2 = 0.98, see table 1 and suppl. figure 4B). For TaPEAMT1 the double reciprocal plot for P-EA fits quite well with the predicted model (data not shown), while for SAM the data points diverge from the expected linear behaviour and appear to fit a parabolic relationship (see figure 2A). The enzyme therefore exhibits strongly cooperative substrate binding for SAM with an apparent specific binding site concentration or n_app of 2 (43). This behaviour is most likely due to the presence of two separate SAM binding sites, as predicted from the presence of two separate methyltransferase domains in the N- and C-terminal halves of the wheat (and spinach) PEAMT enzymes (28,33). For TaPEAMT2 the double reciprocal plots diverge from the expected set of straight lines for both substrates, showing a more hyperbolic pattern, especially when SAM concentration is varied at different constant P-EA concentrations (figure 2B). This indicates negative cooperativity between the two SAM binding sites (43). This behaviour would explain the rather low apparent K_m observed for SAM at physiological substrate concentrations (see above), compared to the huge increase in the K_m for SAM observed at substrate saturation as a result of marked substrate inhibition under these conditions. This might also explain why TaPEAMT2 is 4-fold more active than TaPEAMT1 under a physiological substrate concentration range (200 μM P-EA and 200 μM SAM), while there is only a 2.5-fold difference in V_max between the two enzymes closer to substrate saturation (see table 1).

Product Inhibition – To further elucidate the kinetic mechanisms for both wheat PEAMT isoforms the two reaction products P-Cho and SAH were varied at different fixed concentrations of P-EA and SAM, respectively. The corresponding co-substrate was kept at saturation (figure 3). For TaPEAMT1 apparent K_m values for P-EA and SAM as well as V_max were comparable to the ones obtained from the initial velocity study (see table 1). Both double reciprocal replots show a set of straight lines intersecting above the abscissa and to the left side of the ordinate, pointing to a mixed type of inhibition (see figure 3A+B). The best R^2 values in both studies were obtained for the partial mixed-type inhibition model with values for β > 0 (see table 1 and suppl. figure 4C), indicating that both ESI complexes can still release product. This mixed-type inhibition pattern for both products unequivocally confirms the proposed sequential random Bi-Bi mechanism (43). For
TaPEAMT2 the same pattern of intersecting lines in the double reciprocal plots was observed for both product inhibitors, again confirming a sequential random Bi-Bi mechanism. Values for $\beta$ were very close to 0 in both cases, however, indicating a full mixed-type inhibition that leads to dead-end ESI complexes (see table 1 and suppl. figure 4C). This should allow for a very tight control of TaPEAMT2 activity when either of the two products accumulates. $V_{\text{max}}$ and the apparent $K_m$ for P-\text{EA} are similar to those found in the initial velocity analysis, but the apparent $K_m$ for SAM is significantly lower. Binding of SAH might therefore be the cause of the negative cooperativity observed for TaPEAMT2 in the initial velocity study, leading to an increase in the apparent $K_m$ for SAM. It is quite surprising that the $K_i$ for binding of SAH to the free enzyme is 2-fold higher for TaPEAMT2 than for TaPEAMT1, while $K_i$ values for P-\text{Cho} binding to the free enzyme are almost 3-fold lower for TaPEAMT2 than for TaPEAMT1. Given the more non-competitive nature of TaPEAMT2 inhibition by SAH and the observed negative cooperativity at higher SAM concentrations, it would appear that TaPEAMT2 activity, which is very high at physiologically relevant substrate concentrations, can at the same time be down-regulated very quickly when products accumulate. At substrate saturation TaPEAMT2 is much more sensitive to P-\text{Cho} inhibition than TaPEAMT1 (21 ± 3 % versus 58 ± 2 % of control activity in the presence of 2 mM P-\text{Cho}), as a consequence of its higher affinity for P-\text{Cho} and the strong non-competitive component of this inhibition. Due to the different inhibition mechanisms both isoforms show a similar relative inhibition by SAH despite their contrasting $K_i$ values (56 ± 4 % versus 53 ± 6 % of control activity in the presence of 200 \mu M SAH). For the spinach enzyme an $IC_{50}$ for P-\text{Cho} of 250 to 490 \mu M was reported with a mixed competitive behaviour towards P-\text{EA}, while its sensitivity to inhibition by SAH was high with an $IC_{50}$ of about 10 \mu M (6,33). While both wheat enzymes show an $IC_{50}$ of about 200 \mu M for P-\text{Cho} at 200 \mu M P-\text{EA} and saturating SAM concentrations similar to the spinach enzyme, their $IC_{50}$ for SAH is significantly higher with 70 \mu M for TaPEAMT1 and 130 \mu M for TaPEAMT2 at 300 \mu M SAM and saturating P-\text{EA} concentrations. It therefore seems likely that the wheat enzymes are more robust towards accumulation of SAH in the cell, with TaPEAMT2 being particularly suited to operate at low substrate concentrations.

Effect of Ions and Downstream Metabolites on Wheat PEAMT Activity – The native spinach PEAMT has previously been shown to be sensitive to inhibition not only by P-\text{Cho} and SAH, but also by phosphate, calcium and manganese ions (6). We therefore tested these compounds as well as downstream metabolites choline, GlyBet, PC and PA at substrate saturation (2 mM P-\text{EA} and 4 mM SAM, $n = 2$). 10 mM choline or 50 mM GlyBet had no effect on the two enzymes, with TaPEAMT1 / TaPEAMT2 retaining 96 ± 2 % / 93 ± 3 % and 97 ± 2 % / 102 ± 3 % of control activities, respectively. The activity of both isoforms was mildly repressed by 10 mM potassium (76 ± 9 % / 86 ± 3 %), magnesium (79 ± 9 % / 84 ± 3 %) or calcium phosphate (77 ± 9 % / 84 ± 3 % of TaPEAMT1 / TaPEAMT2 control activity). Manganese ions, however, had a much more drastic effect on the two enzymes: both showed 86 % inhibition at 10 mM MnCl$_2$ (14 ± 5 % / 14 ± 2 % of TaPEAMT1 / TaPEAMT2 control activity), much higher than the 43% inhibition reported for the spinach enzyme (6). Apart from manganese all the other inhibitors tested were less effective than in the spinach PEAMT assay. By far the most effective inhibitor tested in this experiment is PA (figure 4A). The response of the two isoforms is remarkably different. At PA concentrations as low as 100 \mu M TaPEAMT1 activity is already reduced to 33 ± 4 %, while TaPEAMT2 activity is affected only at much higher concentrations. In contrast, even concentrations as high as 1 mM PC do not inhibit either enzyme (see figure 4A), indicating a high degree of specificity and potency of the PA effect on TaPEAMT1 activity.

Binding of PA to Recombinant TaPEAMT Proteins – A number of cytosolic enzymes have been identified as PA binding proteins in eukaryotes (1,44). The binding of PA can result in either inhibition or activation of enzyme activity. As PA was shown to strongly inhibit the PEAMT1 isof orm at low concentration, we wanted to look more closely at the specificity of this interaction using lipid protein overlay assays (41). Figure 4B shows that binding of PA to both isoforms could be detected with as little as 10 pmol PA spotted onto the nitrocellulose membrane and incubation.
with 40 to 80 nM of recombinant protein, while no binding to PC was detected even when spotting as much as 2 nmol PC. In these assays PA binding to TaPEAMT1 appears to be stronger than for TaPEAMT2 (see figure 4B). The specificity of this interaction was examined by analysing the binding of TaPEAMT isoforms to commercially available membrane lipid strips. For both proteins PA was indeed the strongest interacting lipid, but there was also some affinity for phosphatidylinositol (PtdIns)-4-P and to some extent PtdIns(4,5)P₂ and cardiolipin (figure 5A). To see whether the position of the phosphate group within the hexose moiety affects the interaction, commercial PIP strips were incubated with the two recombinant proteins (figure 5B). Again, the strongest interaction by far was observed with PA, followed by PtdIns monophosphates (PtdIns(5)P, (4)P and (3)P) and two of the PtdIns diphosphates (PtdIns(3,4)P₂ and PtdIns(4,5)P₂). Interestingly, no binding could be detected for PtdIns(3,5)P₂ or PtdIns(3,4,5)P₃ suggesting specificity of the recognition. The binding to cardiolipin, but not to PC or other phospholipids, indicates that the spacing of phosphate groups might be important for binding specificity and that it is not merely determined by the size of the ligand. The same holds true for the strong interaction with PA, but not lyso-PA or sphingosine-1-phosphate that merely lack one acyl side chain. To date no lipid binding motif has been identified for PEAMTs and there is not enough sequence similarity between our two PEAMT proteins and published motifs to predict PA or PtdIns monophosphate binding sites. Therefore it has to be assumed that there are yet other binding motifs to be discovered in this protein family.

**DISCUSSION**

Phosphoethanolamine N-methyltransferase catalyses a rate limiting step in plant choline and PC biosynthesis (10,30). Wheat and barley appear to have evolved an alternative pathway for GlyBet accumulation compared to chenopods (5) since they synthesize choline via PC hydrolysis just like GlyBet non-accumulators tobacco and *Arabidopsis* (10,30,44). In the light of this fundamental difference it is important to biochemically characterize PEAMTs from different plant species. The presence of more than one isoform of this enzyme in many species adds a level of complexity to possible regulatory mechanisms that has so far not been explored. Here we report the identification of a second PEAMT isoform in wheat and the comparative biochemical characterisation of both TaPEAMT1 and TaPEAMT2 enzymes. We found that the two genes differ in their expression response to cold exposure in the spring wheat cultivar Egret. An increase in TaPEAMT2 transcript levels was detected in both leaves and roots after 6 days in the cold. TaPEAMT1 gene expression showed no significant variation. In both roots and leaves of unstressed plants TaPEAMT1 transcripts were much more abundant than TaPEAMT2, suggesting that this isoform is likely to be responsible for the bulk turn-over of P-Cho. An increase in total PEAMT protein and activity levels could already be detected within 24 to 48h of transfer to cold, thus preceding TaPEAMT2 gene induction by several days. In *Plasmodium* the rapid reduction in PEAMT protein upon choline addition can be inhibited by bortezomib, a proteasome inhibitor (45). It will therefore be interesting to see whether targeted proteasome-mediated protein degradation or preferential loading of PEAMT mRNA onto the ribosome could explain our observation of a rapid increase in PEAMT protein upon cold treatment ahead of the transcriptional induction of TaPEAMT2.

The present study indicates that on top of this tight control of the absolute protein amount within a given tissue the two differentially expressed wheat PEAMT isoforms also have unique biochemical properties that may be important for plant adaptation to changing environmental conditions: While TaPEAMT1 is less active than TaPEAMT2 and has a lower affinity for one of its substrates, P-EA, it has a higher affinity for the second substrate, SAM, and is less sensitive to feedback inhibition by P-Cho, especially due to the partial nature of that inhibition. This probably makes this enzyme more suitable to maintaining a moderate flux through the pathway under conditions where the methylation index, that is the cellular SAM:SAH ratio (32,46), is high and the cytosolic P-Cho pool builds up to some extent as has been observed in unstressed tobacco leaves with subsequent salinization rapidly depleting this pool (30). The sensitivity of the TaPEAMT1 protein to SAH inhibition means, however, that any disturbance in
the activated methyl cycle will quickly down-regulate this enzyme. In this context it is of note that both PEAMTs have unusually low $K_i$ values for SAH compared to very high $K_m$ values for SAM (20- and 17-fold differences in $K_m$ (SAM) over $K_i$ (SAH) for TaPEAMT1 and TaPEAMT2, respectively) making them very susceptible to inhibition by decreases in the methylating index compared to other methyltransferases (32). It is therefore likely that any increase in PEAMT activity must be accompanied by concomitant increases in activated methyl cycle activities. This coordinated up-regulation has been observed under salt stress for SAM synthetase and PEAMT in the halophyte *Atriplex nummularia* (29) and for SAH hydrolase, adenosine kinase and PEAMT in GlyBet accumulators spinach and sugar beet while there was no evidence for it in the non-accumulators tobacco or canola (32). In the light of the kinetic differences we observed between the two wheat enzymes it is interesting to look at the kinetic mechanisms that have evolved in PEAMT enzymes of other organisms (33,47-49), see also suppl. table 2). Compared to the wheat enzymes the single-domain *Plasmodium* enzyme has a lower specific activity, but higher substrate affinities and is highly sensitive to feedback inhibition by P-Cho. The two separate methyltransferases of *C. elegans* exhibit a random sequential Bi Bi mechanism as the wheat (and spinach) enzymes but are distinctive by their insensitivity to P-Cho inhibition. It may be that the evolution of the two-domain structure of higher plant PEAMTs has facilitated the selection of a moderate sensitivity to P-Cho inhibition, that sits in between that of the highly sensitive *Plasmodium* and the virtually insensitive *C. elegans* proteins.

Given the tight control of PEAMT activity by its products but also the post-transcriptional control most likely exerted by choline ((45,50,51)), we were interested to see whether metabolites further downstream would also act as regulators. It has recently been shown that knock-out of *Arabidopsis NMT1* leads to cell death in the root elongation and differentiation zones that can be reverted by exogenous application of PA (44) and also that cyanobacterial SAM synthetase activity can be stimulated in the presence of PC (52). We therefore tested the effects of these two phospholipids on PEAMT activity. While PC did not affect the *in vitro* activity of the two wheat enzymes, PA led to a very rapid repression of both catalytic activities, with TaPEAMT1 being much more sensitive to PA inhibition than TaPEAMT2. The $IC_{50}$ values obtained for PA inhibition of both enzymes make it likely that repression of TaPEAMT1 activity can occur at physiologically relevant PA concentrations (50 – 150 μM in *Arabidopsis* leaves, (2,53)), while TaPEAMT2 inhibition most likely only occurs under circumstances where endogenous PA levels rise dramatically, as has been demonstrated under hyperosmotic stress and dehydration conditions (54,55). Lipid-protein-overlay studies confirmed the strong specific binding of both PEAMT isoforms to PA but not to glycerolipids, lysolipids, diacylglycerol (DAG) or most other phospholipids. PA signalling has been implicated in numerous plant stress responses (for review see (1)).

The identification of PA as a negative regulator of choline and phospholipid biosynthesis therefore provides a potential mechanism for how plants amplify or attenuate the production of lipid signals. This discovery adds a layer of complexity to the regulation of this biosynthetic pathway since PA and choline are the products of PC hydrolysis by phospholipase D (PLD), and both lead to a rapid down-regulation of PEAMT. Our results indicate that there is a very strong metabolic feedback loop tightly controlling the level of PC synthesis under conditions where rapid turnover of this phospholipid is occurring. In this context it is interesting that wheat PEAMTs also bind to the substrate for PLC hydrolysis, PtdIns(4,5)P$_2$ *in vitro*. In *Arabidopsis* this phospholipid is a potent activator of PLD activity (56), while PLD derived PA is an activator of some PtdIns(4)P 5-kinase isoforms to PA but not to glycerolipids, lysolipids, diacylglycerol (DAG) or most other phospholipids. PA signalling has been implicated in numerous plant stress responses (for review see (1)).

The strongest PIP interaction was observed with PtdIns monophosphates, particularly PtdIns(5)P. This is the most recent member of phosphoinositides detected in plants and it was shown to rapidly accumulate in *Chlamydomonas* cells subjected to hyperosmotic stress alongside its precursor PtdIns(3,5)P$_2$ (58). Interestingly, no binding of the latter to wheat PEAMTs was observed. There was no binding either to PtdIns(3,4,5)P$_3$ (the only phosphoinositide isomer...
that has not yet been detected in plants (1)). Phosphoinositide binding could allow for the recruitment of PEAMT to specific membrane domains ((59,60)) as has been observed for the association of *Plasmodium* PEAMT to the Golgi apparatus (61). The relative contribution of the free phosphobase methylation pathway versus the CDP-choline pathway to *de novo* PC biosynthesis in plants is still a standing question (13). The work in *Plasmodium* suggests that these pathways are not necessarily interchangeable and might lead to structurally different PC pools important for different cellular processes (8). Beyond the present in vitro work it will therefore be essential to examine the regulatory differences between the two wheat PEAMT enzymes in vivo and to gain insights into their contributions to the regulation of PC synthesis and ultimately plant growth and adaptation to stress. Our data open the way for genetic engineering approaches to these questions. Overexpression of a recombinant version of PEAMT that is less sensitive to feedback inhibition by P-Cho has already been discussed as one important tool to increase pool sizes of choline and possibly GlyBet in plants (33). The recent work on two virtually P-Cho insensitive enzymes from *C. elegans* might further assist future engineering efforts (47,48). A challenge will clearly be to overcome the observed strong repression of the PEAMT enzymes by both choline and PA, requiring that the influence of the proteasome on PEAMT stability in plants be addressed and PA binding motifs be identified².
REFERENCES

FOOTNOTES

1 Abbreviations used in this article are: DAG, diacylglycerol; GlyBet, glycine betaine; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; NMT, N-methyltransferase; PA, phosphatidic acid; P-EA, phosphoethanolamine; (Ta)PEAMT, (wheat) phosphoethanolamine N-methyltransferase; PC, phosphatidylcholine; P-Chol, phosphocholine; PIP, phosphoinositides; PtdE, phosphatidylethanolamine; Purity, phosphorylcholylglycerol; Ptdlns, phosphatidylinositol; PtdSer, phosphatidylserine; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosylmethionine.

2 Acknowledgements: We thank Jan Elliot for antibody production and Charles Hocart for helpful discussion. This work has been funded by the Australian National University and the Grains Research and Development Corporation.

FIGURE LEGENDS

Figure 1: PEAMT expression profiles and enzymatic activities in cold acclimated wheat cv. Egret. Seedlings were germinated in sterile vermiculite and watered daily with 1/3 Hoagland solution. They were transferred from 24ºC to 7ºC at the indicated time points and harvested when 2 weeks old. A. Total in vitro PEAMT activity in desalted leaf and root protein extracts (averaged measurements with 70 and 140 μg total leaf protein, left panel, or 20 and 30 μg of total root protein, right panel) after 1, 2 and 6 days of cold exposure (black bar) or continued growth at ambient temperature (open bar) is plotted as mean ± SE nmol SAM / (mg protein * min), n ≥ 3. B. Western analysis of total soluble leaf proteins (10 μg, left panel) and root proteins (4 μg, right panel). A quantification of band intensities relative to controls is given below the α-PEAMT blots. Separation of bands in leaf extracts might be impeded by the RbcL band. Alternatively the second, lower band visible in root extracts could represent an additional PEAMT isoform or hint at a root specific post-translational modification. C. Real-time PCR analysis of TaPEAMT1 (solid bars) and TaPEAMT2 (open bars) expression in cDNA isolated from leaves (left panel) and roots (right panel). Data are relative expression ratios of cold-treated versus control samples, normalised to three control genes (mean ± SE, n = 3).

Figure 2: Initial velocity plots for TaPEAMT1 and TaPEAMT2. Both enzymes follow a sequential random Bi Bi mechanism (R² = 0.975 / 0.981 for TaPEAMT1 / 2). A. Double reciprocal plot of 1/v versus 1/[SAM] for TaPEAMT1 generated at 5 fixed P-EA concentrations of 100 (●), 200 (○), 400 (▼), 800 (□) and 2000 μM (■) using 1 μg of recombinant protein, mean ± SE, n = 2. While plots against 1/[P-EA] are linear (not shown), the plots against 1/[SAM] deviate from a linear relationship (dashed lines) and appear to be parabolic (solid lines). When 1/v is plotted against 1/[SAM]², the linear relationship is restored confirming that PEAMT has two separate binding sites (n_app = 2) which exhibit strong cooperative substrate binding at high [SAM]. B. Double reciprocal plot 1/v versus 1/[SAM] for TaPEAMT2 at 5 fixed P-EA concentrations of 50 (●), 100 (○), 200 (▼), 400 (□) and 1000 μM (■) using 1 μg of recombinant protein, mean ± SE, n = 2. Both double reciprocal plots show a more hyperbolic scattering of data points, with the deviation from a linear relationship being more pronounced for the plot of 1/v against 1/[SAM]. This suggests a partial negative cooperativity of substrate binding, especially at high SAM concentrations. No forcing or weighting of data points was applied.

Figure 3: Product inhibition studies of TaPEAMT1 and TaPEAMT2. A. Double reciprocal plot of 1/v versus 1/[SAM] for TaPEAMT1 using 4 fixed SAH concentrations of 0 (●), 50 (○), 100 (▼), 200 (□) and 500 μM (■), and 1 μg of recombinant protein, mean ± SE, n = 2. B. Double reciprocal plot of 1/v versus 1/[SAM] for TaPEAMT2 using 5 fixed SAH concentrations of 0 (●), 25 (○), 50 (▼), 100 (▼), 200 (□) and 500 (■) μM, and 1 μg of recombinant protein, mean ± SE, n = 2. C. Double reciprocal plot of 1/v versus 1/[P-EA] for TaPEAMT1 using 4 fixed P-Chol concentrations of 0 (●), 1000 (○), 5000 (□), 10000 (■) μM,
and 1 µg of recombinant protein, n = 2. D. Double reciprocal plot of 1/v versus 1/[P-EA] for TaPEAMT2 using 4 fixed P-Cho concentrations of 0 (●), 50 (○), 200 (▽), 500 (□) and 1000 (■) µM, and 1 µg of recombinant protein, mean ± SE, n = 2, with some data points only measured once. No forcing or weighting of data points was applied.

**Figure 4:** PA inhibition of TaPEAMT activity and PA binding to TaPEAMT isoforms. A. *In vitro* activity of recombinant TaPEAMT1 (circles) and TaPEAMT2 (squares) in the presence of increasing PA (solid symbols) or PC (open symbols) concentrations, expressed as percent of activity in control reactions in the absence of lipids. Reactions contained 1 µg of recombinant protein and saturating substrate concentrations (2 mM SAM and 4 mM P-EA), mean ± SE, n ≥ 2. While the presence of PC vesicles does not alter the activity of either enzyme, addition of PA vesicles lead to a rapid decrease in TaPEAMT1 activity with an IC₅₀ of about 70 µM PA, while TaPEAMT2 is only inhibited by very high PA concentrations with an IC₅₀ of about 470 µM PA. B. Specificity of PA binding. 80nM recombinant TaPEAMT protein was incubated with membranes spotted with concentration series for PC and PA, followed by decoration with primary and secondary antibodies and chemiluminescence detection. Bound TaPEAMT protein can be detected with as little as 10 pmol PA spotted, while PC does not attract any protein to the membrane, even with as much as 2 nmol spotted. A representative blot selected out of 3 membranes for each PEAMT isoform in 2 independent experiments is shown. Numbers below chemiluminescence signals represent the average spot intensity across replicated membranes expressed as % of signal intensity obtained for TaPEAMT1 at the maximal PA concentration spotted.

**Figure 5:** Screen of putative lipid ligands for the two TaPEAMT isoforms. A. Membrane lipid strips spotted with 100 pmol of each lipid were incubated with 80 nM recombinant TaPEAMT protein as before. As expected both isoforms strongly interact with PA, followed by some binding to PtdIns(4)P > PtdIns(4,5)P₂ / cardiolipin. Numbers next to each chemiluminescent signal are given as % of spot intensity of PA binding to TaPEAMT1. B. In order to test the specificity of the interaction with phosphoinositides PIP strips (spots contain 100 pmol lipid each) were incubated with 80 nM recombinant TaPEAMT protein as before. The strongest interaction again is observed with PA, followed by interactions with the three PtdIns monophosphates ((5)P > (4)P > (3)P) and two of the PtdIns diphosphates ((3,4)P₂ ≥ (4,5)P₂) consistent with earlier findings, except that TaPEAMT2 binding to PtdIns(4,5)P₂ was not detected in A most likely due to a more stringent wash. The recognition appears to be specific as no binding to PtdIns, PtdIns(3,5)P₂ or PtdIns(3,4,5)P₃ was observed. Numbers next to each chemiluminescent signal describe spot intensities expressed as % of spot intensity for PA binding to TaPEAMT1.
Table 1: Summary of the kinetic parameters obtained from the biochemical analysis of the two wheat PEAMT proteins. Data were fitted to equations (1) to (3) given in suppl. figure 4A that describe the sequential random Bi Bi (suppl. figure 4B) and partial / full mixed inhibition mechanisms (suppl. figure 4C).

<table>
<thead>
<tr>
<th>Initial velocity study</th>
<th>TaPEAMT1</th>
<th>TaPEAMT2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P-EA [µM]</strong></td>
<td>100 – 2000</td>
<td>50 – 1000</td>
</tr>
<tr>
<td><strong>SAM [µM]</strong></td>
<td>200 – 4000</td>
<td>100 – 4000</td>
</tr>
<tr>
<td><strong>Vmax [nmol/(mg*min)]</strong></td>
<td>552±29</td>
<td>1351±82</td>
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<tr>
<td><strong>Km(app., P-EA) [µM]</strong></td>
<td>390±120</td>
<td>185±49</td>
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<tr>
<td><strong>Km(app., SAM) [µM]</strong></td>
<td>601±178</td>
<td>1,060±295</td>
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<tr>
<td><strong>α</strong></td>
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<td>1.2</td>
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<td><strong>mechanism</strong></td>
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<tr>
<td><strong>R² (n=2)</strong></td>
<td>0.975</td>
<td>0.981</td>
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**P-Cho feedback inhibition**

| **P-EA [µM]**          | 100 – 4000 | 200 – 4000 |
| **SAM [µM]**           | 2000 | 4000 |
| **P-Cho [µM]**         | 1000 – 10000 | 50 – 1000 |
| **Vmax [nmol/(mg*min)]** | 430±12 | 1231±35 |
| **Km(app., P-EA) [µM]** | 328±38 | 263±34 |
| **Ki(P-Cho) [µM]**     | 306±57 | 137±38 |
| **α**                  | 5.5      | 2.5      |
| **β**                  | 0.32     | —        |
| **mechanism**          | mixed    | mixed    |
| **R² (n=2)**           | 0.971    | 0.975    |

**AdoHyc feedback inhibition**

| **P-EA [µM]**          | 2000 / 4000 | 2000 |
| **SAM [µM]**           | 300 – 4000 | 300 – 4000 |
| **SAH [µM]**           | 50 - 500 | 25 - 500 |
| **Vmax [nmol/(mg*min)]** | 436±24 | 1495±63 |
| **Km(app., SAM) [µM]**  | 655±112 | 754±96 |
| **K(SAH) [µM]**       | 31±8 | 70±16 |
| **α**                  | 3.5      | 5.7      |
| **β**                  | 0.47     | 0.07     |
| **mechanism**          | mixed    | mixed    |
| **R² (n=2)**           | 0.943    | 0.966    |
FIGURES

figure 1

A

B

C

in vivo PEAMT activity

control 1d 2d 6d

control 1d 2d 6d

cold stress
α-PEAMT 1 2.5 2.9 6.2

cold stress
α-PEAMT 1 0.8 1.4 7.2

α-Rbcl

α-TCP

rel. expression [log2]

1d 2d 6d

1d 2d 6d

control 1d 2d 6d

control 1d 2d 6d

rel. expression [log2]
**figure 2**

(A) 

(B)
**Figure 4**

**A**

![Graph showing activity as a percentage of control over a range of concentrations.](image)

**B**

![Images showing TaPEAMT1 and TaPEAMT2 with PC and PA levels at different concentrations.](image)
**figure 5**

A

![A sample image with various chemical compounds and their concentrations](image)

B

![B sample image with various chemical compounds and their concentrations](image)
Biochemical characterization of two wheat phosphoethanolamine N-methyltransferase isoforms with different sensitivities to inhibition by phosphatidic acid

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